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Merocrine secretion from serous cutaneous glands in *Rana esculenta* complex and *Rana iberica*

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Serous cutaneous glands of larval and juvenile specimens pertaining to the *Rana esculenta* complex and *Rana iberica* may modulate product discharge through exocytotic release and weak compression from the myoepithelial sheath. This method of secretory release is a typically merocrine mechanism and is an unusual functional trait in these glands, which are generally credited with holocrine bulk discharge of anti-predatory poison products. Current trends in animal toxinology claim that the most common active compounds in anuran poisons (i.e. biogenic amines and peptides) played an early role in skin homeostasis during the phylogeny of this order, before participating in chemical skin defence against predators. On the basis of this hypothesis, we conclude that the merocrine activity described in *Rana* serous cutaneous glands is an ancestral functional characteristic related to the use of skin products in local regulative mechanisms.

INTRODUCTION

The concept of holocrine secretory activity performed by serous glands in anuran skin was developed by FARAGGIANA (1938*b*), who carried out pioneering experimental studies on the regenerative processes which follow glandular discharge (FARAGGIANA, 1938*a*, 1939). Her findings were later confirmed under TEM (DELFINO, 1980), and further investigations, coupled with pharmacological approaches (BENSON & HADLEY, 1969; HOLMES et al., 1977; HOLMES & BALLS, 1978; DELFINO et al., 1982), stressed the role of the myoepithelial cells in the massive release of the serous product together with the secretory cytoplasm and nuclei.

The latter method of secretory discharge is consistent with the use of the serous secretory products as anti-predatory toxins and/or repellents (DUELLMAN & TRUEB, 1985). Nonetheless, recent studies on the *Rana esculenta* complex, showing morphological changes in the granules containing serous skin products, suggested that these substances are also employed in skin homeostasis (BARNI et al., 1987) Furthermore, ultrastructural evidence has proved that discrete amounts of product are released by serous glands in juvenile *Hyla arborea* through exocytosis (DELFINO et al., 1994). These findings disclose

new perspectives to the interpretation, both functional and evolutive, of serous skin secretion in anurans (DALY et al., 1987). In the light of this updated approach to the regulating function of poison secretions of anuran skin, we aimed to investigate whether the serous cutaneous glands of two groups of European frogs, the green frogs (Italian *Rana esculenta* complex) and the brown frogs (Spanish *Rana iberica*), can release their cutaneous serous products through exocytosis, i. e. by merocrine mechanisms. The above taxa were studied as they represent a genus which includes extensively studied species, both morphologically and physiologically, commonly found in laboratories. This choice enabled us to obtain adequate references from previous literature and at the same time allows verification of our results.

We avoided any conditions which could elicit bulk secretion and focused on the patterns which could refer to the modulated release of the serous product. We observed advanced larval specimens and newly metamorphosed froglets, as these development stages appeared to best fit our purpose. Serous glands in late tadpoles show advanced patterns of biosynthesis but relatively undifferentiated myoepithelial cells, a condition which may *minimize* bulk discharge; on the other hand, juveniles are very imperfect terrestrial vertebrates and must *maximize* their cutaneous homeostatic mechanisms when exposed to the subaerial environment.

We detected evidence of merocrine secretory processes and compared them with the traditional holocrine mechanism reported in serous cutaneous glands of anurans.

MATERIAL AND METHODS

Larval specimens of Italian green frogs (*Rana esculenta* complex) and of the Iberian brown frog *Rana iberica* were collected from the outskirts of Florence (Italy) and Mellid (Santiago de Compostela, Spain), respectively. The tadpoles were reared in the laboratory until the first specimens underwent metamorphosis. The schedule below reports the developmental range considered, according to TAYLOR & KOLLROS (1946), as well as the number of animals and the samples observed for each, under light (LM) and electron microscopes (TEM).

(A) Stage XX, *Rana esculenta* complex: LM, 3 tadpoles, 2 samples from each; TEM, 2 tadpoles, 2 samples from each.

(B) Stage XXII, *Rana iberica*: LM, 3 tadpoles, 3 samples from each; TEM, 3 tadpoles, 3 samples from each.

(C) Stage XXV (juvenile), *Rana esculenta* complex: LM, 3 froglets, 2 samples from each; TEM, 2 froglets, 2 samples from each.

(D) Stage XXV, (juvenile) *Rana iberica*: LM, 2 froglets, 2 samples from each; TEM, 2 froglets, 2 samples from each.

The choice of the developmental range was based on former studies which showed that, between stages XIX and XXV, anuran skin possesses small, but already differentiated glands which already contain large secretory accumulations undergoing post-Golgian maturation (DELFINO, 1977; DELFINO et al., 1988, 1994). Furthermore, this developmental

range also embraces the differentiation timing of the glandular myoepithelial sheath (DELFINO et al., 1987), which plays a role in gland depletion.

Tadpoles and froglets were anaesthesized in 0.2 % aqueous chlorbutol and sacrificed. Small bands of skin (about 4 mm^2) were removed from the back and fixed in Karnovsky's aldehyde mixture (KARNOVSKY, 1965). The skin strips were then reduced into smaller (about 2 mm^2) fragments and post-fixed in 1 % OsO_4. For all these procedures, a 0.1 M, pH 7 sodium cacodylate buffer was employed, at a temperature of 4°C. The specimens were dehydrated in a graded ethanol series, soaked in propylene oxide and infiltrated with Epon 812 to obtain flat embeddings. The embeddings were then cut with a NOVA LKB ultramicrotome into semithin (1-1.5 µm) and ultrathin (silver-white interference colour) sections, these sections were used for the light and electron microscope observations, respectively. The semithin sections were stained with buffered 10 % toluidine and used to prepare ultrastructural investigation. The ultrathin sections were collected on uncoated copper grids and stained with a hydroalcholic saturated solution of uranyl acetate, followed by bismuth subnitrate (0.8 mg/ml in an alkaline solution). These specimens were finally observed under a Siemens 101 electron microscope at 80 kV.

RESULTS

Under the light microscope, serous glands at premetamorphic stages XX and XXII (fig. 1a-b) exhibit the structural feature characteristic of the Anura. The secretory units, which are syncytial in structure, are provided with a continuous contractile sheath of myoblasts (the future myoepithelial cells, mec) as well as a cap of undifferentiated cells, representing the regenerative matrix (intercalary tract or neck) of the gland. According to the usual ultrastructural patterns shared by all serous glands of anurans, the poison adenomeres of *Rana* tadpoles possess exiguous lumina, which are restricted to the subintercalary level. However, in advanced larvae of the Iberian frog, these cavities are rather enlarged (fig. 1a-b) when compared to other anurans studied so far. The luminal space sinks into the secretory syncytium and resembles a multichambered compartment, owing to the interposition of slender cytoplasmic walls, which look like thin bridges between the secretory unit and neck (fig. 1a-b). Observation of serial sections revealed that duct and gland lumina are separated only by a discontinuous cytoplasmic screen (fig. 1b). Actually, the intercalated cells, which are arranged in an irregular doughnut structure, partly obstruct the central opening with slender cytoplasm projections. The secretory product may reach the duct lumen through the spaces separating these cell processes. Secretory granules crowd around the luminal boundary and mould themselves around its surface, so that they assume a crescent-like shape in section.

In juvenile glands, the serous syncytium is a solid structure, thus limiting the small lumen to the neck region (fig. 1c). The secretory cytoplasm holds large amounts of poison product, which consists of dense particles, subspherical in shape However, larger magnifications revealed a spongy-like substructure in some granules (fig. 1d).

Under the electron microscope, the secretory syncytium possesses a well developed biosynthesis apparatus and nuclei scattered in a single row at the periphery. Slender rough

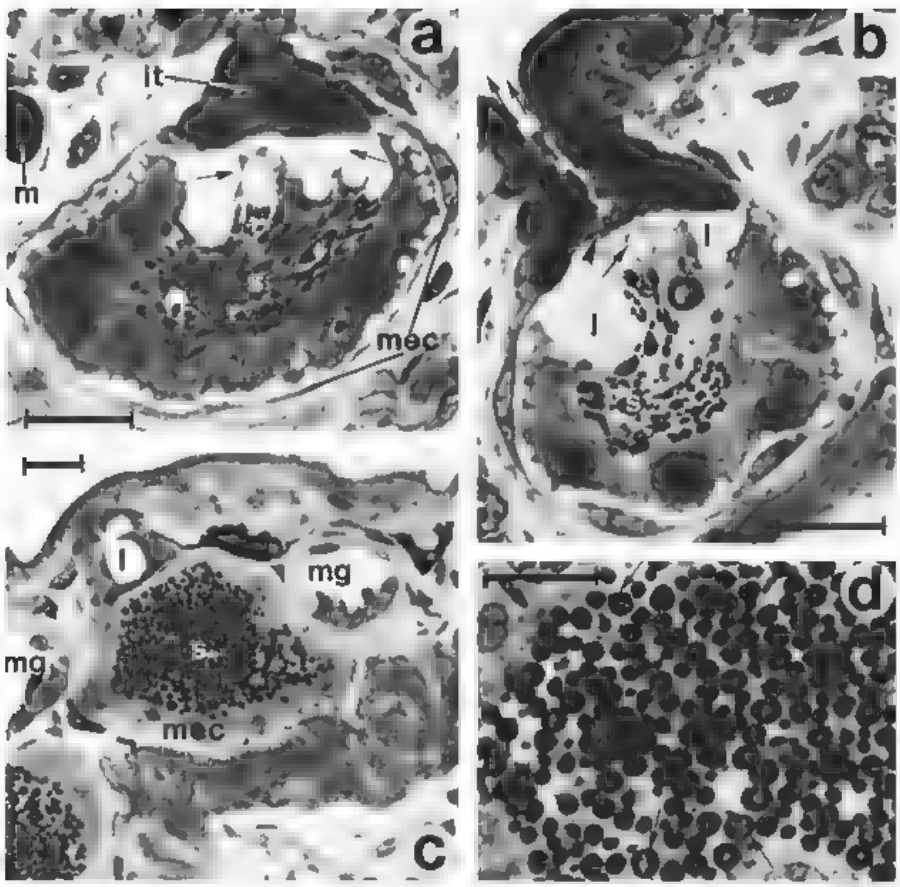

Fig. 1. — Serous gland anlagen under light microscope. Bars: 10 µm.

a. — *Rana iberica* (XXII). Longitudinal section tangential to the intercalary tract (it), consisting of a few layers of undifferentiated cells The apical portion of the secretory syncytium displays a multichambered lumen with slender cytoplasmic partitions (arrows). Notice the serous secretory product (s) crowded around the lumen and the thin myoepithelial cell layer (mec). m: melanophore.

b. *Rana iberica* (XXII). Longitudinal section through the widest diameter of the secretory unit, as shown by the lumen of the duct which is obvious Only a thin, discontinuous screen (arrowhead), formed by slender cytoplasmic processes of intercalary tract cells, separates the duct and adenomere lumina Coupled arrows in the duct outlet, arrow points to cytoplasmic partition between apical chambers. s: serous secretory product.

c. — *Rana esculenta* complex (juvenile) Longitudinal section the secretory unit lacks any obvious lumen, a slender cavity (l) is detectable only in the intercalated tract-duct complex. Note two mucous glands (mg) provided with a wide lumen mec· myoepithelial cell layer; s· serous secretory product

d. *Rana esculenta* complex (juvenile). Detail of the serous secretory product contained in the syncytial cytoplasm. Arrows point to granules with a spongy-like substructure.

endoplasmic reticulum (rer) cisterns (fig. 2a-b) and Golgi stacks (dictyosomes) (fig. 2a, 2c-d) are consistent in both species observed, and occupy the peripheral cytoplasm. The distal (trans) face of the Golgi stacks dispatches minute vesicles (fig. 2d), which merge together to form wide, up to 4 μm in diameter, secretory deposits. These large vesicles contain a finely dispersed product (fig. 2c-e, 5a), which undergoes marked condensation (maturation) in later stages of biosynthesis (fig. 3a). Several merging processes affect the secretion aggregates (fig. 3b, 4), which sometimes display a spongy-like substructure, owing to the alternation of darker and paler zones. Secretory product maturation is a gradual process in which the granules become involved at different rates. The process produces a remarkable polymorphism, due to the coexistence of intermediate stages of condensation. In some instances the intermediate steps are by-passed, as revealed by condensation patterns affecting the material freshly dispatched from the Golgi stacks (fig. 3c).

During the condensation phase, the membrane which borders the serous aggregates becomes detached from the secretory material and leaves a transparent halo around it In the meantime, numerous, slender (about 25 nm in diameter), microvilli-like outgrowths of the cytoplasm intrude this perigranular space. The microvilli are branched and hollow, and form a network around the secretory product (fig. 3d).

From the central cytoplasm the secretory granules reach the upper level of the syncytium, just beneath the lower cell layer of the intercalary tract (neck) of the gland. As observed under light microscope, a proper lumen exists at this level, separated from the neck and duct lumina only by interposition of slender cytoplasmic processes of the intercalated tract cells. The serous deposits crowd around the lumen (fig. 4) and some adhere to the luminal plasmalemma, so that their limiting membranes merge with it. Rather large openings form in this way, which allow the secretory product to be released into the lumen (fig. 4, inserts A and B), following the pattern of a merocrine process. This mechanism appears, however, to be quite peculiar; the spaces holding the granules engaged in the secretory release are continuous with the compartments containing other serous aggregates, due to serial confluences (fig. 4, insert B). In this way the secretory product may flow out toward the lumen, at a rate which depends on the number of granules involved in reciprocal confluence.

The secretory units engaged in this exocytotic activity possess the neuro-contractile apparatus typical of serous glands, consisting of neurites and myoepithelial cells (fig 5a). The thin axons are clearly recognizable as they contain parallel neurotubules (fig. 5b-d) and electron transparent vesicles in their endings (fig. 5c), whereas in the myocytes myofilaments occupy large portions of the cytoplasm, leaving two symmetrical zones at the nuclear poles to accommodate scanty organelles (fig. 5a). Nevertheless, this muscle-nerve cell machinery seems to be still immature, since myofilaments fail to fill the myocyte cytoplasm, and neuromuscular junctions are infrequent and resemble poorly specialized, occasional contacts (fig. 5b). In their erratic course, some neurites may also make contact with the secretory syncytium (fig. 5d).

Serous glands in recently metamorphosed froglets display large amounts of cytoplasm filled with dense aggregates, which arise from further condensation of the secretory deposits described in larval adenomeres. Despite their density, secretory granules are not homogeneous in aspect as they exhibit the spongy-like substructure (fig. 6a-b) already

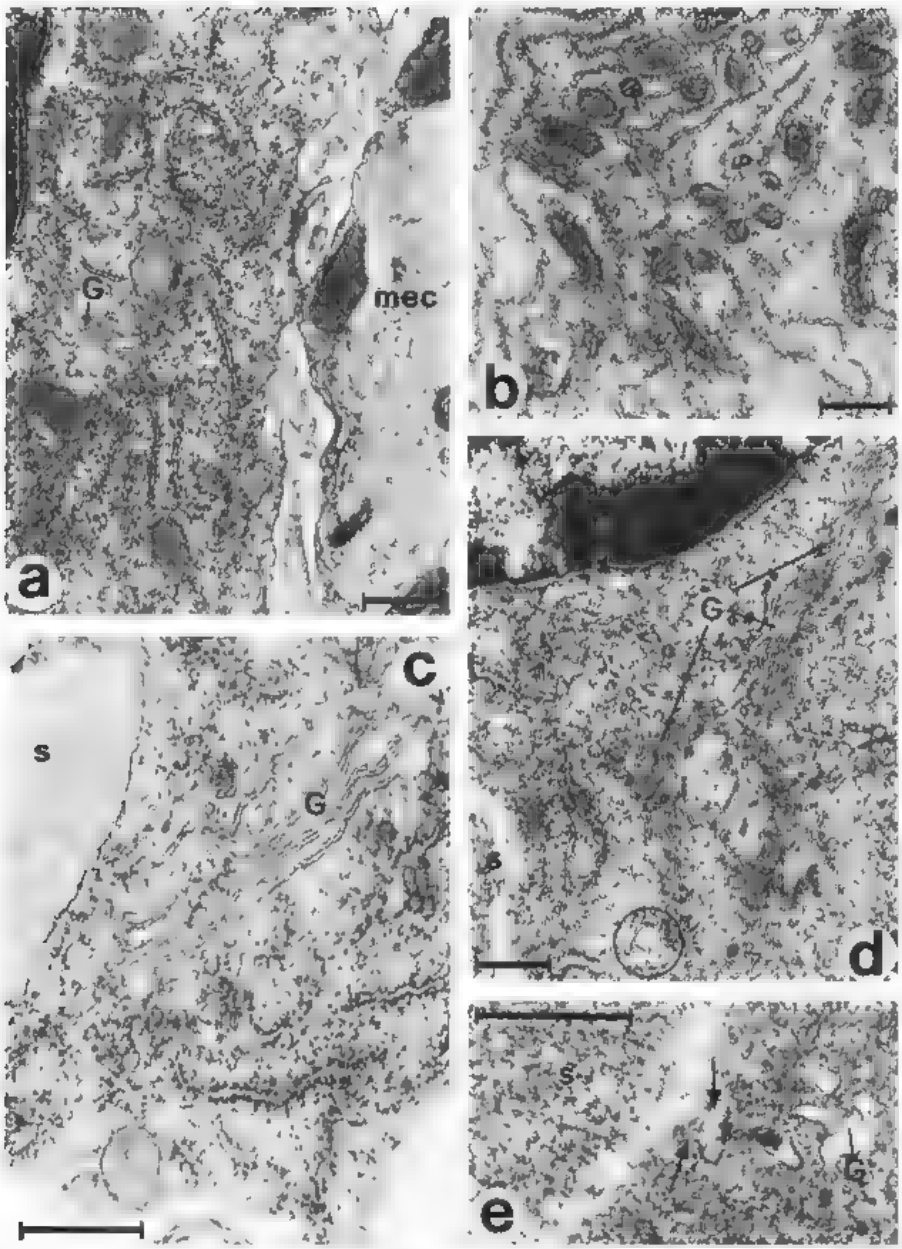

Fig 2 – Biosynthesis apparatus in serous gland anlagen of both species. Bars 500 nm

a – *Rana esculenta* complex (XX). Peripheral portion of the secretory syncytium, closely contiguous to the myoepithelial layer. Note slender rer cisterns and a Golgi stack, or dictyosome (G). mec: myoepithelial cell.

b *Rana iberica* (XXII) Parallel array of rough endoplasmic reticulum complements and mitochondria with dense matrix

c. – *Rana esculenta* complex (XX). Golgi stacks (G) frequently occur in the perinuclear cytoplasm of the secretory syncytium The dictyosomes fulfil the activity of the rough endoplasmic reticulum and lead to the accumulation of a fine serous secretory material (s) inside large vesicles.

d-e – *Rana iberica* (XXII) Minute vesicles (encircled in d) derive from the periphery of the Golgi stacks (G) and merge (arrows in e) with the larger serous secretory deposits (s), contributing material to these storage structures.

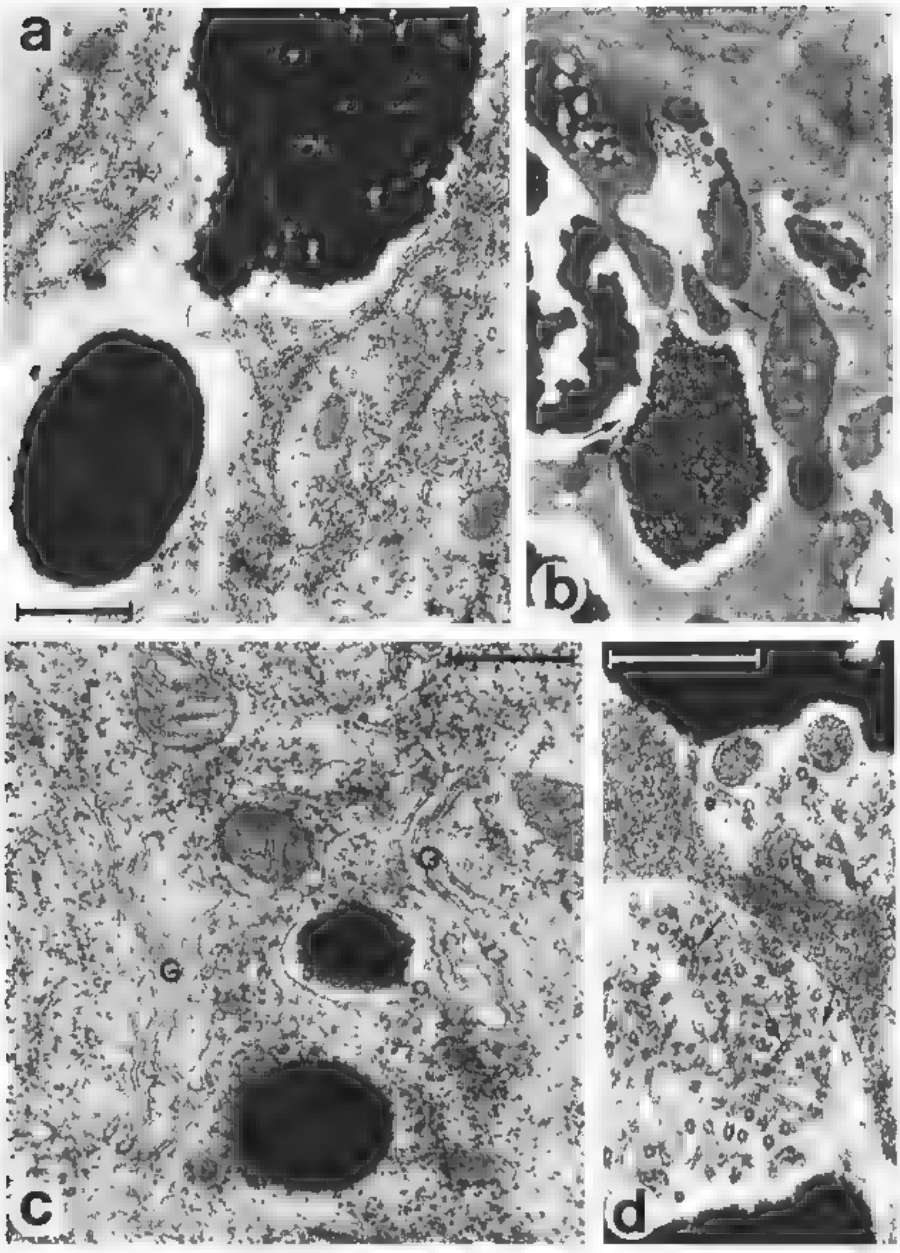

Fig. 3. – Patterns of poison maturation in both species. Bars: 500 nm.

a. – *Rana esculenta* complex (XX). The secretory product contained in the vesicles undergoes a remarkable condensation, which gives rise to granules provided with a compact substructure

b. – *Rana iberica* (XXII). Serous maturation, recognizable from variable density of the product, proceeds through sequential confluence processes between secretory aggregates (arrows).

c. – *Rana esculenta* complex (XX). In some instances the product contained in the vesicles dispatched by the Golgi stacks (G) is promptly condensed and the vesicle phase is by-passed

d. – *Rana iberica* (XXII). Slender microvilli fill the space between the limiting membrane and secretory product Arrows indicate branching points which give the microvillous cluster the appearance of a three-dimensional net.

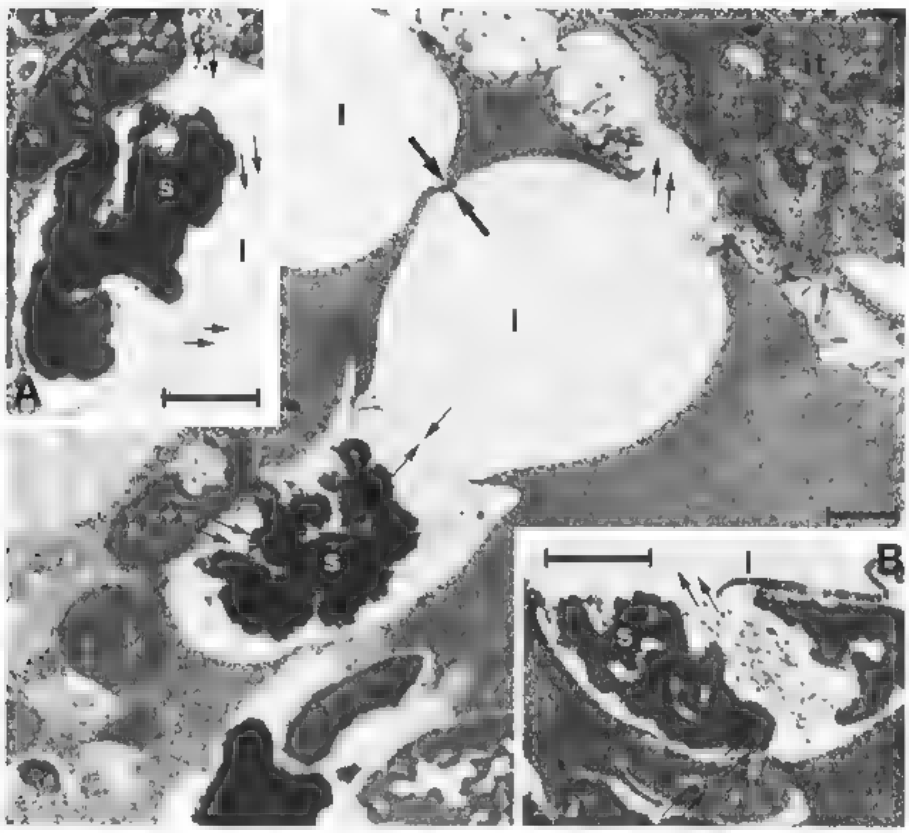

Fig. 4. — *Rana iberica* (XXII). Bars: 1 µm. Subintercalar portion of serous secretory unit Note the multichambered lumen (l), the characteristic cytoplasmic walls (large arrows) and secretory aggregates of various density. Only an extremely thin screen (small arrows) separates the large vesicle from the lumen. Inserts A and B show continuity between vesicular and luminal compartments and between serous vesicles (B). Coupled arrows show outflow paths. it intercalary tract; s' serous secretory product.

described in the previous stages. In neometamorphosed froglets, the biosynthesis apparatus typical of protein manufacturing glands decays remarkably owing to rarefaction of rer and dictyosomes The organelles are first reduced to the very perinuclear zone and later disappear altogether (fig. 6a-b).

Touch stimulation of cutaneous areas, possibly painful in nature, may evoke local responses in the myoepithelial sheath of serous glands before sacrifice. Contracted myocytes show well-defined thickenings in their myofilament apparatus, which in turn cause remarkable morphological changes in the shape of the nuclei. Myofilaments act as a sphincter around the surface of the nucleus and mould it into the shape of an hourglass, with the peripheral half contained in the myocyte and the inner one bulging towards the secretory syncytium (fig. 6c).

The effects elicited by myocyte compression may be confirmed by patterns detectable in the gland duct. This is a slender intraepidermal interstice that crosses the epithelial

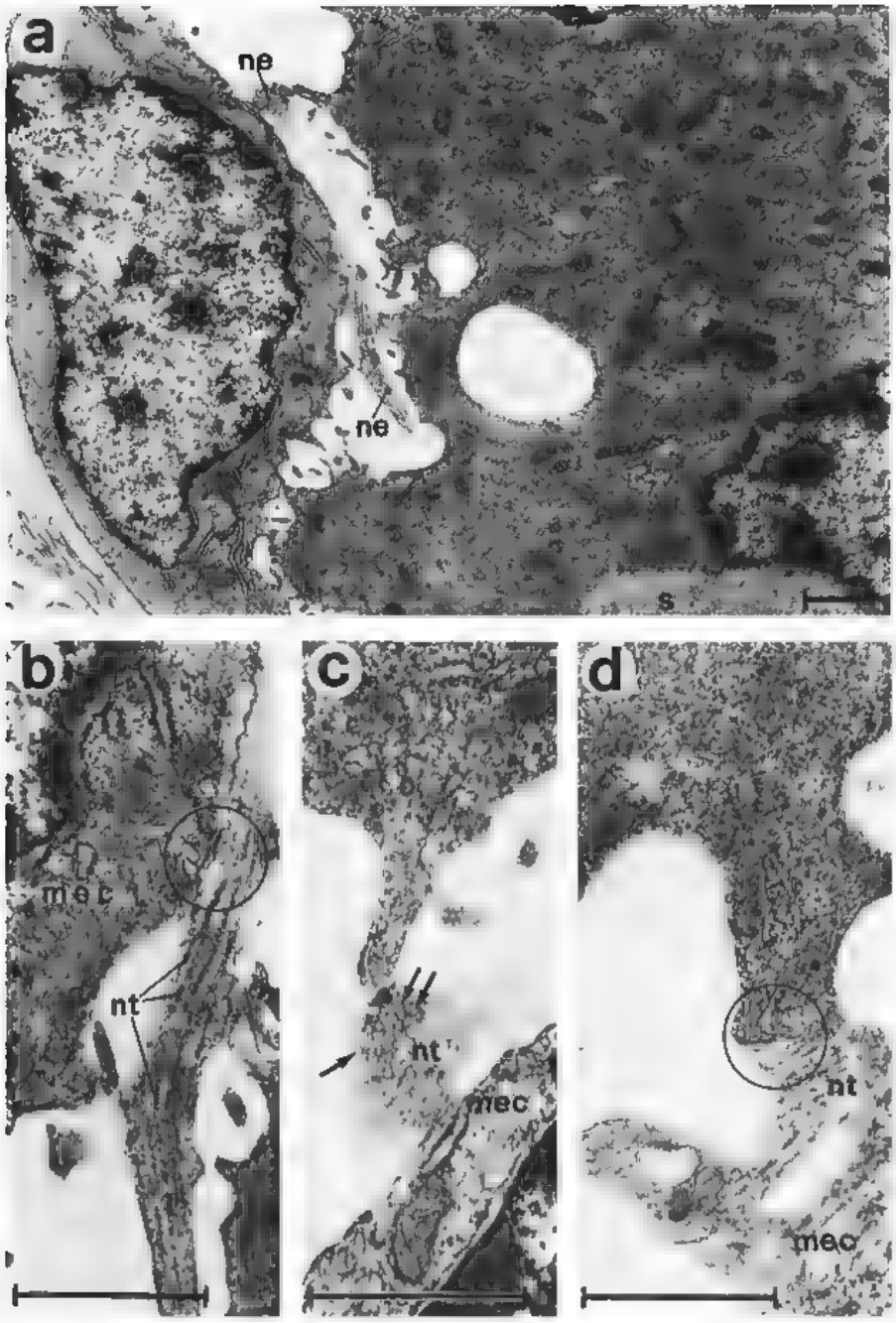

Fig. 5. — *Rana iberica* (XXII). Bars: 1 μm. The neuromuscular apparatus of the serous gland anlagen consists of myoepithelial cells (mec) and neurites (ne). nt: neurotubules, s. serous secretory product

a — Note the dome-shaped, perinuclear portion of the myoepithelial cell engaged in myofilament accumulation. The biosynthesis apparatus is obvious at the cell poles Neurites are contained in the interstice between secretory and contractile compartments.

b. — Detail of the previous figure. Note parallel neurotubules in the axon and non-specialized contacts between neurite and myoepithelial cell (encircled).

c. — Detail of (a) showing a neurite ending. Small electrontransparent vesicles (arrows) are obvious

d — In some instances, only a 30 nm wide gap (encircled) separates the neurite from the serous syncytium.

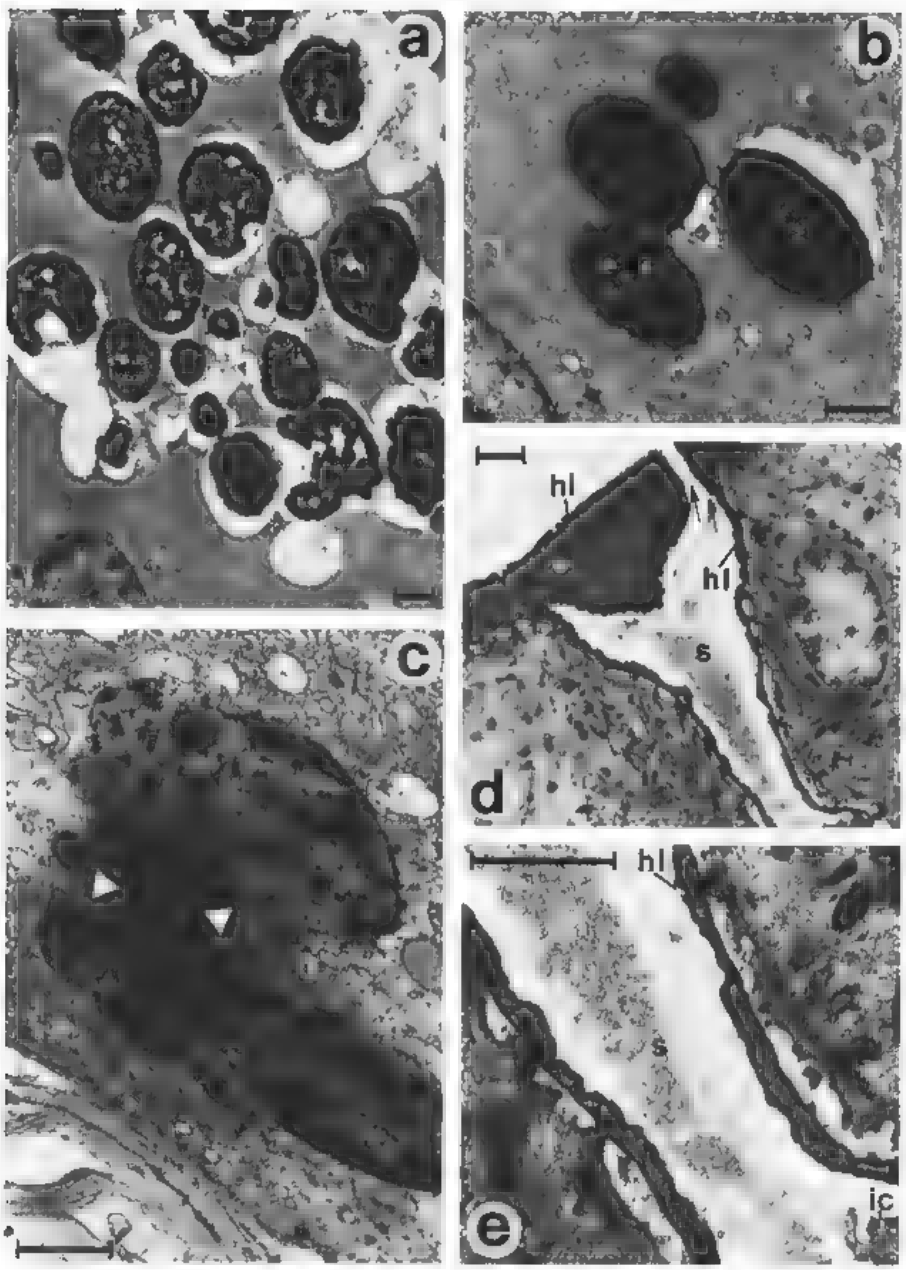

Fig. 6 – Serous glands in juveniles. Bars: 1µm.

a-b. – *Rana iberica* (a) and *Rana esculenta* complex (b): the serous product consists of granules with spongious substructure and high electrondensity; the secretory organules are restricted to the syncytium periphery.

c. – *Rana esculenta* complex in contracted myoepithelial cells the nuclei bulge toward the secretory syncytium, arrowheads indicate a dense ring of myofilaments around the nucleus which is shaped like an hourglass.

d. *Rana esculenta* complex: gland duct reaching the body surface, lined with horny layer (hl) and keratinocytes. The serous secretory product (s) consists of a structureless, moderately electrondense material; coupled arrows indicate duct outlet

e. Enlargement of the previous micrograph, showing a detail of the duct; only cytoplasmic projections of intercalary tract cells (ic) are interposed between duct and neck lumina. s serous secretory product, hl: horny layer.

layers, bounded by a horny lining, continuous with the external *stratum corneum* of the body surface (fig. 6d). The duct lumen contains a rather opaque, structureless material, devoid of any membranous body (fig. 6e). The lack of membrane bounded serous material within the lumen is consistent with merocrine secretory release.

DISCUSSION AND CONCLUSIONS

Several ultrastructural and pharmacological investigations have depicted the discharge mechanism in serous cutaneous glands in anurans. The interstices between the secretory unit and myoepithelium hold axonal endings (BÖCK & LERTPRAPAI, 1972; WHITEAR, 1974) which form synaptic junctions with the myocytes and contain dense-cored vesicles (150 nm in diameter), adrenergic in appearance (DOCKRAY & HOPKINS, 1975; SJÖBERG & FLOCK, 1976; DELFINO, 1979, 1991; BARBERIO et al., 1987). This sympathetic innervation has been confirmed by nor-epinephrine stimulation which allowed pharmacological characterization of receptors on myoepithelial cell membranes (BENSON & HADLEY, 1969; HOLMES et al., 1977; HOLMES & BALLS, 1978; DELFINO et al., 1982). TEM observations disclosed typical ultrastructural features in still contracted mec: myocytes show alternating thickenings among myofilaments, whereas their nuclei are displaced towards the secretory syncytium; the plasmalemma facing the stroma displays several folds (DELFINO, 1980, 1991; DELFINO et al., 1987, 1990). In the Italian green frog specimens, we observed both myofilament thickenings and nuclear displacements, although the patterns were not so dramatic as in experimental specimens The serous product is stored within the syncytium cytoplasm, so that the intense compression exerted by the myoepithelial sheath, triggered by pharmacological stimulation, causes bulk discharge of the secretory product, syncytium cytoplasm and nuclei (DELFINO, 1980). When the secretory product consists of dense particles, they can be collected in saline and were found to still possess their limiting membranes (DOCKRAY & HOPKINS, 1975, in *Xenopus laevis*). This excludes that granule release occurs through exocytosis, which involves insertion of the membrane encompassing the secretory particle into the plasmalemma. Under pharmacological stimulation, the activity of serous cutaneous glands of anurans may be regarded as *holocrine* in nature, since it involves emission of fragments of the secretory units. This bulk emission mechanism, which requires phasic contractions achieved through neuromuscular machinery, is well consistent with the defensive role ascribed to the serous cutaneous glands of anuran skin.

However, recent trends credit these secretory units, at least in *Rana* species, to perform a regulatory role in the water balance of the skin. MILLS & PRUM (1984) assign this function to specialized cells of the mucous and *seromucous* glands (the latter represent a novel type found by these authors in *Rana catesbeiana*, *Rana pipiens* and *Rana temporaria*), whereas in the *Rana esculenta* complex BARNI el al (1987) stressed the role of the secretion (*venom*) of large serous glands in controlling water balance. This is suggested by changes both in granule morphology (vacuolization) and chemical composition, fitting the annual cycle of the frog. The cycle includes two periods (activity, hibernation) with alternating changes in skin permeability. The above authors detected a

Tal mecanismo de secreción contrasta con la función defensiva, antipredatoria, del veneno de los anuros, basado sobre un proceso holocrino; sin embargo, el mecanismo merocrino es coherente con la ancestral función reguladora atribuida a algunos componentes de esta secreción cutánea

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